PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Searching for a link between the L-BMAA neurotoxin and Amyotrophic Lateral Sclerosis: study protocol of the French BMAALS program
AUTHORS	Delzor, Aurélie; Couratier, Philippe; Boumédiène, Farid; Nicol, Marie; Druet-Cabanac, Michel; Paraf, François; Méjean, Annick; Ploux, Olivier; Leleu, Jean-Philippe; Brient, Luc; Lengronne, Marion; Pichon, Valérie; Combès, Audrey; El Abdellaoui, Saïda; Bonneterre, Vincent; Lagrange, Emmeline; Besson, Gérard; Bicout, Dominique; Boutonnat, Jean; Camu, William; Pageot, Nicolas; Juntas-Morales, Raul; Rigau, Valérie; Masseret, Estelle; Abadie, Eric; Preux, Pierre-Marie; Marin, Benoît

VERSION 1 - REVIEW

REVIEWER	Marco Vinceti Creagen- University of Modena and Reggio Emilia Medical School, Italy
REVIEW RETURNED	30-May-2014

GENERAL COMMENTS	This is an interesting manuscript since it clearly and extensively reports the protocol of a research project which may be of considerably help to understand if one (cyanotoxins), or more environmental risk factors are actually involved in the putative environmental etiology of ALS. In particular, this manuscript highlights both the strengths and the limitations of the protocol of a large and innovative study currently undergoing in France. Though publication of a study protocol is not always worth doing, in the present case this may be of considerable interest for the several investigators working in the same field, and to foster a discussion about methodological issues of such an approach. Of particular interest are also the evaluation of a cyanobacterial proliferation model based on environmental and microbiological data, and the comparison of L-BMAA quantification methods in different areas of the world (as reported in Figure 3). I have no major issue and limitations to outline about this manuscript. On the converse, I have some minor points to highlight: - Some mention of the data analysis approaches which the authors plan to use, if any, would be of interest, focusing on spatial data analysis and related statistics - Will the authors use any kind of spatial modeling to assess and predict the cyanotoxins dispersion and distribution around the potential point sources (e.g., water bodies)? - It would be useful to give an estimate of how large are the single areas under studies (districts and counties, page/lines=10/54-61 and 11/1-2) and the related populations - ALS etiology is a process which likely takes years if not decades to

	develop, and therefore geocoding ALS cases (and of the population controls) at time of diagnosis may be inadequate to identify potential environmental factors based on proximity to pollution sources, particularly if the residential mobility of the underlying population is high. The authors should asses and discuss the potential relevance of this issue and their methodological implications, and/or add the identification of residential history to the study protocol - Might it be useful to add 'French' before 'BMAAL program', or 'in France' after 'program', in the Title?

REVIEWER	Tracie Caller Dartmouth-Hitchcock Medical Center, Lebanon New Hampshire, USA I am engaged in similar exploration of BMAA as an environmental risk factor of ALS.
REVIEW RETURNED	09-Jun-2014

GENERAL COMMENTS	this article represents a steady protocol submitted for review, to further explore the possible connection of ALS and the cyanobacteria neurotoxin BMAA. This is a large, ambitious study with many separate components, including geospatial analysis for a spatial association between the toxin, cyanobacteria blooms and ALS development, as well as a case control component. The authors have also incorporated some methodology to try to examine for the presence of the BMAA in both the environment as well as tissues of ALS patients (and controls). Comments regarding the methodology: Overall, it appears that the authors comprise a strong team, and have good retrospective case data to work with. In the Hypothesis, I would clarify that the geographic analysis explores spatial association (not cause-and-effect); I agree that it could potentially estimate potential cyanobacterial exposure in combination with patient history about prior exposures, and I believe that's what the others are trying to convey that it needs to be a bit more clear. There is mention of exploration of cyanobacterial dietary supplements but I did note in the method section this was not described in detail.' The availability of adequate retrospective data is a strength; I also appreciate that these be captured recapture method which will help reduce the potential for false positive cases. They may want to comment if there is potential for missed diagnoses (for example, seen by general neurologist but not referred to an ALS specialist) or incorrect diagnoses. On page 9, lying for-5, the "levels" a data analysis are bit confusing to me, as is page 10 sentence 15. The geographic statistics mentioned on page 10 line 54-55 are not explained in detail. It would be helpful for the authors to describe how they plan on incorporating the maps of ALS patients with the cyanobacterial maps, and what statistics exactly will be used. It would also be helpful if they can clarify the methodology for this and no bacteria blooms. I presume they will combine the various data to determine some sort of severity of cyanobacteria exposure for each water body, but this is not clearly described. This is retrospective ascertainment of environmental exposure by patient and family member report is of course subject to recall bias. I

	am not clear how feasible or reliable it will be to obtain history from family members have a patient who died in 2003. The authors may want to consider a prospective study. Should third should clarify whether the questionnaire the use will be developed and tested by their research group, or if they are planning on using established questionnaires. Food consumption in particular will be very difficult to assess retrospectively. A food diary recall for the past 24 or 72 hours might be possible, but again I am not clear how a family member might be able to recall dietary history from the patient who died many years ago. In addition, dietary habits may change after the diagnosis of ALS in patients with bulbar symptoms who had difficulty swallowing. I am also a little unclear about whether the choice of controls is adequate. I appreciate the inclusion of testing tissue samples for BMAA as I think this is critical to exploring this hypothesis further. Comments regarding the paper: - Introduction of the nice overview of the BMAA hypothesis. The first and second paragraphs could potentially could be shortened, I do not believe a substantial amount of time needs to be spent in describing genetic mutations. In paragraph 2, there are also other environmental and occupational exposures that have been postulated and the list provided in sentence 27-30 may not be all inclusive. In paragraph 3, the other should certainly mention the work of Dunlop et al (later mentioned in the paper) as this would provide a mechanism for chronic exposure to low levels of BMAA, while many of the animal models assessed acute toxicity. Page 4 sentence 23-31 does not seem entirely relevant to the topic on hand and could be omitted, instead the authors may want to expand on why BMAA detection is controversial. The authors may also also want to mention the previous work done in France with regards to spatial clustering in the introduction as it strengthens there objective in hypothesis. - Methods: The overview of the protocol is helpful in guiding the reader through a very elaborate study. Some portions of the method section, for example page 12 lines 29-34 might be more appropriate for the introduction of background rather than the method section. - Discussion: Age 13, line 23, the authors may also want to mention that underlying genetic predisposition may also be at play. Lines 36-40, again the authors should be sure to mention the work of Dunlop et al as there is another potential mechanism for BMAA toxicity through incorporation into tRNA. Lastly, I would recommend a sentence or 2 as a conclusion, summarizing why it is important that their work should be pursued despite the limitations.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: Marco Vinceti
Institution and Country: Creagen- University of Modena and Reggio Emilia Medical School, Italy
Please state any competing interests or state ‘None declared’: None declared

Manuscript bmjopen-2014-005528 titled “Searching for a link between the L-BMAA…”
This is an interesting manuscript since it clearly and extensively reports the protocol of a research

project which may be of considerably help to understand if one (cyanotoxins), or more environmental risk factors are actually involved in the putative environmental etiology of ALS. In particular, this manuscript highlights both the strengths and the limitations of the protocol of a large and innovative study currently undergoing in France. Though publication of a study protocol is not always worth doing, in the present case this may be of considerable interest for the several investigators working in the same field, and to foster a discussion about methodological issues of such an approach. Of particular interest are also the evaluation of a cyanobacterial proliferation model based on environmental and microbiological data, and the comparison of L-BMAA quantification methods in different areas of the world (as reported in Figure 3).

I have no major issue and limitations to outline about this manuscript. On the converse, I have some minor points to highlight:

- Some mention of the data analysis approaches which the authors plan to use, if any, would be of interest, focusing on spatial data analysis and related statistics

Answer: According to this remark, we have clarified which type of geostatistical analyses will be used (page 11).
“Geostatistical analyses, based on Kulldorff statistics, will be performed to identify areas of significant over- or under-incidence as compared to the referral incidence value, which is the global incidence in the whole area under study.”

- Will the authors use any kind of spatial modeling to assess and predict the cyanotoxins dispersion and distribution around the potential point sources (e.g., water bodies)?

Answer: A paragraph has been added to answer this question (page 11-12).
“Geographic statistics will be then performed in order to classify each administrative unit (e.g. municipality) according to four parameters: i) the number of days of sunshine, ii) temperature, iii) the area of stagnant water (included dam and ponds) and iv) data on P and N withdrawal. For the last one, anthropogenic factors will also be considered as industrial and agricultural activities can impact on N and P release (use of organophosphorus compounds, for example). This multi-criteria approach will allow obtaining an index of promoting cyanobacterial blooms. The same will be done with watersheds as there is an aggravating effect from upstream to downstream of P and N inputs. Finally, a coefficient correlation will be measured between SIR and the calculated index.”

- It would be useful to give an estimate of how large are the single areas under studies (districts and counties, page/lines=10/54-61 and 11/1-2) and the related populations

Answer: We have answered this question with the following table (page 8).
Mean population (2003-2011) PYFU
LIMOUSIN
Corrèze 239,630 2,156,666
Creuse 123,179 1,108,607
Haute-Vienne 368,404 3,315,632
LANGUEDOC-ROUSSILLON
Hérault 1,007,451 9,067,055
Pyrénées-Orientales 433,243 3,899,187
RHÔNE-ALPES
Ardèche 307,119 2,764,067
Drôme 471,348 4,242,128
Isère 1,175,146 10,576,314
Savoie 404,247 3,638,219

Haute-Savoie 707,077 6,363,693
TOTAL 5,236,844 47,131,568

- ALS etiology is a process which likely takes years if not decades to develop, and therefore geocoding ALS cases (and of the population controls) at time of diagnosis may be inadequate to identify potential environmental factors based on proximity to pollution sources, particularly if the residential mobility of the underlying population is high. The authors should asses and discuss the potential relevance of this issue and their methodological implications, and/or add the identification of residential history to the study protocol

Answer: Indeed, this point is crucial in our study and we have considered it. For reflecting that, we have modified the paragraph as follows (page 13).
“As there is probably a long latency period between exposure and appearance of ALS 135-136 and given that L-BMAA exists in a protein-associated form which could act as an endogenous neurotoxic reservoir over time 54, in-depth study will involve gathering details of dwelling since birth (in order to precise their residential history), and for other items from the age of 13. Indeed, French population is always in movement (Figure 4) and it has to be considered in our study.”

- Might it be useful to add ‘French’ before ‘BMAAL program’, or ‘in France’ after ‘program’, in the Title?

Answer: New title of the study protocol is now: “Searching for a link between the L-“BMAA neurotoxin and Amyotrophic Lateral Sclerosis: study protocol of the French BMAALS program”.”

I would suggest some minor changes to the manuscript, particularly concerning one methodological details and potential limitations, and how to minimize the latter ones. However, I believe that this manuscript is really worth considering for publication.

Reviewer: 2
Reviewer Name: Tracie Caller
Institution and Country: Dartmouth-Hitchcock Medical Center, Lebanon New Hampshire, USA
Please state any competing interests or state ‘None declared’: I am engaged in similar exploration of BMAA as an environmental risk factor of ALS.

This article represents a steady protocol submitted for review, to further explore the possible connection of ALS and the cyanobacteria neurotoxin BMAA.
This is a large, ambitious study with many separate components, including geospatial analysis for a spatial association between the toxin, cyanobacteria blooms and ALS development, as well as a case control component. The authors have also incorporated some methodology to try to examine for the presence of the BMAA in both the environment as well as tissues of ALS patients (and controls).

Comments regarding the methodology:

Overall, it appears that the authors comprise a strong team, and have good retrospective case data to work with.

- In the Hypothesis, I would clarify that the geographic analysis explores spatial association (not cause-and-effect); I agree that it could potentially estimate potential cyanobacterial exposure in combination with patient history about prior exposures, and I believe that's what the others are trying to convey that it needs to be a bit more clear.

Answer: We have taken into account these remarks and change the sentence as follows (page 6-7).

“ii) of geo-epidemiology to investigate patients’ environment (dwelling, occupational and leisure) in order to assess spatial association (not cause-and-effect) between ALS cases and a putative cyanobacterial exposure in combination with patient history about prior exposures.”

- There is mention of exploration of cyanobacterial dietary supplements but I did note in the method section this was not described in detail.

Answer: Cyanobacterial dietary supplements are questioned through an item of the questionnaire and asking for precisions when necessary. This has been added into the manuscript (page 12).
“iii) food consumption whom dietary supplements and if any, the type of supplement is informed.”

- The availability of adequate retrospective data is a strength; I also appreciate that these be captured recapture method which will help reduce the potential for false positive cases. They may want to comment if there is potential for missed diagnoses (for example, seen by general neurologist but not referred to an ALS specialist) or incorrect diagnoses.

Answer: This important point has been more precisely described (page 9).
“For the case ascertainment, we founded our methodology on three sources: i) ALS referral centers from France, ii) health insurance data and iii) hospitals. It was not possible, while tempted, to involve private neurologists because it was not possible for them to retrieve retrospective information about past ALS patients seen in their practice (problems of lack of computerized database). Hence, we relied on these three sources only. This methodology has been applied in the FRALim register 99 (first register of ALS in France, located in Limousin, for the period 2000-2011). The case ascertainment was also based on these three sources and we estimated, thanks to capture-recapture analysis, an exhaustiveness of the register of 98.4% (95% CI 95.6-99.4), thus a low number of false negative cases 99 (ie. missed cases). As for the other departments in the BMAALS project we applied the same methodology, we expect the same high level of exhaustiveness.”

- On page 9, lying for-5, the "levels" a data analysis are bit confusing to me,

Answer: To be clearer, we have added one figure (page 10, figure 3).

- as is page 10 sentence 15.

Answer: Sentence has been modified (page 10).
“Thus, those imprecise definitions do not explain clearly what a cluster is: how many cases do we need for considering having a cluster?”

- The geographic statistics mentioned on page 10 line 54-55 are not explained in detail. It would be helpful for the authors to describe how they plan on incorporating the maps of ALS patients with the cyanobacterial maps, and what statistics exactly will be used. It would also be helpful if they can clarify the methodology for this and no bacteria blooms. I presume they will combine the various data to determine some sort of severity of cyanobacteria exposure for each water body, but this is not clearly described.

Answer: As it has been also remarked by reviewer 1, we have modified the paragraph (page 11-12).
“Geographic statistics will be then performed in order to classify each administrative unit (e.g. municipality) according to four parameters: i) the number of days of sunshine, ii) temperature, iii) the area of stagnant water (included dam and ponds) and iv) data on P and N withdrawal. For the last one, anthropogenic factors will also be considered as industrial and agricultural activities can impact on N and P release (use of organophosphorus compounds, for example). This multi-criteria approach will allow obtaining an index of promoting cyanobacterial blooms. The same will be done with

watersheds as there is an aggravating effect from upstream to downstream of P and N inputs. Finally, a coefficient correlation will be measured between SIR and the calculated index."

- This is retrospective ascertainment of environmental exposure by patient and family member report is of course subject to recall bias. I am not clear how feasible or reliable it will be to obtain history from family members have a patient who died in 2003. The authors may want to consider a prospective study.

Answer: The program has been designed as a retrospective study and has already been accepted. So, we can't change now our strategy but we will consider this remark for a future program.

- Should third should clarify whether the questionnaire the use will be developed and tested by their research group, or if they are planning on using established questionnaires.

Answer: A sentence has been added (page 12).
"The questionnaire has been developed by the consortium specifically for the BMAALS program."

- Food consumption in particular will be very difficult to assess retrospectively. A food diary recall for the past 24 or 72 hours might be possible, but again I am not clear how a family member might be able to recall dietary history from the patient who died many years ago.

Answer: In ALS, it is suspected that there is a long-term incubation. So, a 24 or 72 hours diet recall will not reflect what we are looking for. We have added references which show the reliability of food frequency questionnaire on a long period (from 8 to 24 years) (page 14). Nonetheless, the bias of interviewing relatives' patients is real and except by being as clear as possible, we did not found any other alternatives.
"Food frequency questionnaires for long-term recall (from 8 to 24 years) show to be reliable 138-142: they appear to be a good alternative to food diary recall for diseases with a potential long-term incubation."

- In addition, dietary habits may change after the diagnosis of ALS in patients with bulbar symptoms who had difficulty swallowing.

Answer: During interview, we precise that we want to know dietary habits before diagnosis and first symptoms (page 14). Moreover, we have also developed a self-administered questionnaire administrated prospectively from 2012 in another research study (ancillary study).
"To avoid any misinterpretation of the question concerning dietary habits, it is clearly clarified that it concerns habits before diagnosis and first symptoms. Moreover, we also have developed a self-administered questionnaire given to all ALS patients (not only those included to our program) and we will compare answers between patients since 2012 and those from 2003-2011 (ancillary study)."

- I am also a little unclear about whether the choice of controls is adequate.

Answer: Controls are matched on sex, age, city and should not have any neurological diseases (clarified page 12). This is what is routinely done in case-control studies.
"Controls will be matched on age at diagnosis, sex, city and will should not present any neurological pathologies."

I appreciate the inclusion of testing tissue samples for BMAA as I think this is critical to exploring this hypothesis further.

Comments regarding the paper:

- Introduction of the nice overview of the BMAA hypothesis. The first and second paragraphs could potentially could be shortened, I do not believe a substantial amount of time needs to be spent in describing genetic mutations.

Answer: Paragraph 1 (page 5) has been shortened.
“But since, others mutations 3-8 have been discovered whom C9orf72 (chromosome 9 open reading frame 72), TARDBP (TDP-43 encoding gene) and FUS (Fused in Sarcoma protein) are commonly identified in FALS cases 8-16.”

- In paragraph 2, there are also other environmental and occupational exposures that have been postulated and the list provided in sentence 27-30 may not be all inclusive.

Answer: Indeed, we have not listed all potential exposures, only the more often cited (page 5).
“Another controversial hypothesis, often cited, is that physical activity, whether occupational or leisure-related, is a risk factor for SALS 38-42.”

- In paragraph 3, the other should certainly mention the work of Dunlop et al (later mentioned in the paper) as this would provide a mechanism for chronic exposure to low levels of BMAA, while many of the animal models assessed acute toxicity.

Answer: It has been done (page 6, reference 68).

- Page 4 sentence 23-31 does not seem entirely relevant to the topic on hand and could be omitted, instead the authors may want to expand on why BMAA detection is controversial.

Answer: Sentence has been removed. BMAA detection controversy is already discussed (page 15).

- The authors may also also want to mention the previous work done in France with regards to spatial clustering in the introduction as it strengthens there objective in hypothesis.

Answer: Indeed, Masseret et al. show that BMAA is found in France. Reference has been added (page 6, reference 83).

- Methods: The overview of the protocol is helpful in guiding the reader through a very elaborate study. Some portions of the method section, for example page 12 lines 29-34 might be more appropriate for the introduction of background rather than the method section.

Answer: This paragraph has been included in the introduction (page 6).

- Discussion: page 13, line 23, the authors may also want to mention that underlying genetic predisposition may also be at play.

Answer: It has been added (page 15).
“iii) genetic predisposition may also be at play 145.”

- Lines 36-40, again the authors should be sure to mention the work of Dunlop et al as there is another potential mechanism for BMAA toxicity through incorporation into tRNA.

Answer: It has been added (page 15).
“iv) association to protein due to mischarging of tRNA 68.”

- Lastly, I would recommend a sentence or 2 as a conclusion, summarizing why it is important that their work should be pursued despite the limitations.

Answer: We have followed this recommendation (page 17).
"Despite of limitations mainly lying on bias due to interviews of patients' relatives and the controversy on BMAA analysis, this program is of importance because it is the first to investigate the cyanobacteria hypothesis in France."

VERSION 2 – REVIEW

REVIEWER	Marco Vinceti Department of Diagnostic, Clinical and Public Health Medicine, University of Modena and Reggio Emilia, Modena, Italy
REVIEW RETURNED	14-Jul-2014

- The reviewer completed the checklist but made no further comments.

REVIEWER	Tracie Caller Adjunct Instructor in Neurology Geisel School of Medicine at Dartmouth Hanover, NH, USA I am a co-investigator on a non-federal grant examining BMAA as an environmental risk factor for ALS.
REVIEW RETURNED	23-Jul-2014

GENERAL COMMENTS	Overall, this paper describes the methodology for a very arduous research proposal. Overall, this revision is improved from the prior document and the overall scope is more clearly defined. Abstract page 5 - Line 14: change to "Using multiple sources" - Line 25: clarify if drinking water; "brain tissue" rather than brain. Page 7, line 27: would reword this sentence to "frequent head trauma, and contact with certain chemicals such as pesticides, formaldehyde, and organic solvents." Delete "contact with pesticides" in line 26. Page 8 line 18: "…concentration, more recently it was shown…." Page 8 line 48: change putative to potential Page 9 line 30: how will you assess patients who do not garden? I would assume not everyone raises their own fruits and vegetables. Page 9-10 case ascertainment: the authors need to clarify who approved the study since they did obtain identifying information about patients. They briefly mention this in the abstract but need to reiterate in the methods. Page 11 3rd paragraph: too lengthy and cumbersome, would reword to say "This methodology of case ascertainment using the same 3 sources has been previously applied in the FRALim register 100 (first register of ALS in France, located in Limousin, for the period 2000-2011), and we estimated, thanks to capture-recapture analysis, an exhaustiveness of the register of 98.4% (95% CI 95.6-99.4), yielding a low number of false negative cases 100. Data from private neurologists were not obtained because most lacked computerized records and a retrospective chart review was not feasible.

	Regarding the methods, I understand that this project is a large undertaking and it is impossible to describe all methods in detail. However, the methodology regarding the spatial analysis remains a bit murky and perhaps is not presented at the level that it could easily be replicated. Consider referencing the methodology of other studies. That being said, I realize that this is a proposal and that the actual study has not yet been conducted. The statistical tests comparing overexposed vs underexposed cohorts is not well defined (what is a significant difference?) and I am not clear if each environmental factor will be analyzed separately or using a regression model. Have the authors considered incorporating a time component in these maps, similar to the prior work of Sabin et al? Page 13 line 52-57: I am not exactly clear how this data will be used, particularly data regarding "all plant species". This seems overwhelming to incorperate. Page 14 line 1-3: how will sampling water for cyanobacteria add to the hypothesis? It could possibly be helpful to know which species are identifiable in the water bodies, but how will you know if the conditions reflect the water body 10, 20, or 50 years prior which is the likely exposure window? Lack of cyanobacteria/BMAA in 2014 does not mean that it was not present 20 years ago. Core sediment sampling may be of greater use. Page 14 line 43: will the questionnaire be included as an appendix or otherwise be available for review? Was it based on an existing, validated questionnaire (i.e. Stanford ACES modules) or has it otherwise been validated by the authors? Page 16 line 22: how is this known?

VERSION 2 – AUTHOR RESPONSE

Reviewer Name: Tracie Caller
Institution and Country: Adjunct Instructor in Neurology
Geisel School of Medicine at Dartmouth
Hanover, NH, USA
Please state any competing interests or state 'None declared': I am a co-investigator on a non-federal grant examining BMAA as an environmental risk factor for ALS.

Overall, this paper describes the methodology for a very arduous research proposal. Overall, this revision is improved from the prior document and the overall scope is more clearly defined.

Abstract page 5
- Line 14: change to "Using multiple sources"
Answer: The sentence has been modified (page 3).
"Using multiple sources of ascertainment,…"

- Line 25: clarify if drinking water; "brain tissue" rather than brain.
Answer: These two points have been modified (page 3).

“Specimens of drinking water, food and biological material (brain tissue) will be examined…”

Page 7, line 27: would reword this sentence to “frequent head trauma, and contact with certain chemicals such as pesticides, formaldehyde, and organic solvents.” Delete “contact with pesticides” in line 26.
Answer: The sentence has been reworded (page 5).
“Other associations have been proposed as occupational exposure to electromagnetic fields 23 26-29, frequent head trauma 34-35, contact with certain chemicals such as pesticides, formaldehyde, organic solvents and heavy metals 23 30-33 36-37.”

Page 8 line 18: “…concentration, more recently it was shown….”
Answer: The sentence has been modified (page 6).
“First of all, L-BMAA was found to be produced by a wide range of cyanobacteria 55-56 66-67 71-73; more recently it was shown that diatoms, the most common group of algae, could also produce it 74.”

Page 8 line 48: change putative to potential
Answer: The word has been changed (page 6).
“Furthermore, a case-control study will be performed to investigate the potential routes of contamination by L-BMAA…”

Page 9 line 30: how will you assess patients who do not garden? I would assume not everyone raises their own fruits and vegetables.
Answer: For patients who have a garden, sampling of water and food will be easy. For the others, we will look for vegetables purchase from a local producer, if any. But, we keep in mind that the L-BMAA hypothesis is suitable for some patients but could not explain all cases (page 7).
“Collection of drinking water, fruits and vegetables from patients who garden…”

Page 9-10 case ascertainment: the authors need to clarify who approved the study since they did obtain identifying information about patients. They briefly mention this in the abstract but need to reiterate in the methods.
Answer: A sentence has been added (page 8).
“After obtaining authorizations from CCTIRS (Comité Consultatif sur le Traitement de l’Information en matière de Recherche dans le domaine de la Santé) and CNIL (Commission Nationale de l’Informatique et des Libertés), nominative data are obtained from the French national coordination of ALS referral centers, public and private hospitals in the areas of interest, and health insurance data related to long duration diseases.”

Page 11 3rd paragraph: too lengthy and cumbersome, would reword to say “This methodology of case ascertainment using the same 3 sources has been previously applied in the FRALim register 100 (first register of ALS in France, located in Limousin, for the period 2000-2011), and we estimated, thanks to capture-recapture analysis, an exhaustiveness of the register of 98.4% (95% CI 95.6-99.4), yielding a low number of false negative cases 100. Data from private neurologists were not obtained because most lacked computerized records and a retrospective chart review was not feasible.
Answer: Paragraph has been modified (page 9).
“This methodology of case ascertainment using the same three sources that has been previously applied in the FRALim register 100 (first register of ALS in France, located in Limousin, for the period 2000-2011). We estimated, thanks to capture-recapture analysis, an exhaustiveness of the register of 98.4% (95% CI 95.6-99.4), yielding a low number of false negative cases 100 (ie. missed cases). Data from private neurologists were not obtained because most lacked computerized records and a retrospective chart review was not feasible.”

Regarding the methods, I understand that this project is a large undertaking and it is impossible to

describe all methods in detail. However, the methodology regarding the spatial analysis remains a bit murky and perhaps is not presented at the level that it could easily be replicated. Consider referencing the methodology of other studies. That being said, I realize that this is a proposal and that the actual study has not yet been conducted.
Answer: We have already detailed the analysis and some definitions in the paper after the first revision. It seems difficult to develop further at this stage as the reviewer has recognized. We will follow in particular the methods detailed in Boumediene et al. 2011 (ref 26) and we have referenced this paper at the end of the dedicated paragraph, as proposed by the reviewer.

The statistical tests comparing overexposed vs underexposed cohorts is not well defined (what is a significant difference?) and I am not clear if each environmental factor will be analyzed separately or using a regression model.
Answer: To compare overexposed and underexposed cohorts, we will use classical statistical tests (t-tests or Mann-Whitney tests for continuous variables and Chi-square or Fisher exact tests for nominal variables) considering the “a priori” fixed level of risk. Obviously, we will do both analyzes, univariable and multivariable for each environmental factors.

Have the authors considered incorporating a time component in these maps, similar to the prior work of Sabin et al?
Answer:Time component is a complex issue.
We have only a period of 11 years of inclusion of patients. It will then be a too short period to search for temporal or spatio-temporal clustering.
With a low survival rate in this disease, we will have only few patients (about 50) investigated by questionnaires. Obviously, in these questionnaires we will include an attempt to estimate the exposition duration in particular using the residential history. So we think that we will have a low power to analyze this component.

Page 13 line 52-57: I am not exactly clear how this data will be used, particularly data regarding “all plant species”. This seems overwhelming to incorporate.
Answer: We will investigate in particular plants with industrial wastes with a high phosphorus level because they favor the development of cyanobacterial blooms in the environment.

Page 14 line 1-3: how will sampling water for cyanobacteria add to the hypothesis? It could possibly be helpful to know which species are identifiable in the water bodies, but how will you know if the conditions reflect the water body 10, 20, or 50 years prior which is the likely exposure window? Lack of cyanobacteria/BMAA in 2014 does not mean that it was not present 20 years ago. Core sediment sampling may be of greater use.
Answer: We have clarified this point (page 12).
“Gathering information about favorable conditions for cyanobacterial blooms will allow us to model their expansion notably in term of meteorology and nutrient inputs. In addition to the use of previous collected data, we will be able to know if there were cyanobacteria prior to patients’ diagnosis, which species and so if there was a risk of L-BMAA presence in water.”

Page 14 line 43: will the questionnaire be included as an appendix or otherwise be available for review? Was it based on an existing, validated questionnaire (i.e. Stanford ACES modules) or has it otherwise been validated by the authors?
Answer: Questionnaire has specifically been developed for the BMAALS program and is not based on an existing and validated questionnaire. Validity of the questionnaire has not been yet determined.

Page 16 line 22: how is this known?
Answer: A sentence has been added to answer this question (page 14).

“Likewise, due to the fact that ALS is a rare disorder, areas of significant under-incidence are characterized by absence or almost absence of patients”